—Technical Paper—

Construction and Characterization of Glucose Enzyme Sensor Employing Engineered Water Soluble PQQ Glucose Dehydrogenase with Improved Thermal Stability

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In this study, we constructed glucose enzyme sensors with increased thermal stability employing engineered PQQGDH-B. First, we demonstrated the enzyme glucose sensor employing an engineered PQQGDH-B, Ser231Lys. The residual activity after heat treatment at 60℃ for 2 hours of the enzyme electrode employed Ser231Lys was 80% of the initial activity, whereas the electrode employed native PQQGDH-B was 30%. Second, we investigated the effect of cross-linking chemical modification on the thermal stability of PQQGDH-B. The cross-linked PQQGDH-B had high thermal stability and the half life time at 55℃ was 63 min whereas that of native enzyme was 4 min. Furthermore, we demonstrated the Flow Injection Analysis (FIA) system for glucose measurement employed the cross-linked PQQGDH-B. The high operational stability of this system showed the very stable responses (SD was within 3%) for 100 times of glucose injection.

Key Words : Glucose Dehydrogenase, Pyrroloquinoline Quinone, Thermal Stability, Glucose Sensor

1 Introduction

Various glucose dehydrogenases (GDHs) were reported to possess pyrroloquinoline quinone (PQQ) as their prosthetic group. PQQGDHs have been given extensive attention as enzyme sensor constituents, because of their property of being oxygen independent.[1,2] Although PQQGDHs have such superior properties, further improvement of the enzymatic properties is being required, considering and comparing with those of the most popular and major enzyme utilized for glucose sensors, glucose oxidase, *e.g.* substrate specificity and operational stability. Two types of PQQGDHs have been reported; the membrane binding single peptide PQQGDH (PQQGDH-A), and the water soluble dimeric PQQGDH (PQQGDH-B). Focusing on the high homology within PQQGDH-As, the authors have been attempting to identify the protein region responsible for each enzymatic characteristic using extensive homology analyses together with mutational analyses.[3-9] We also reported that by cross-linking chemical modification, PQQGDH-A thermal stability was significantly enhanced.[10] Since PQQGDH-B loses its activity by the dissociation of quaternary structure, cross-linking chemical modification of PQQGDH-B will also provide the stabilized enzyme. We previously reported on the construction of several engineered PQQGDH-Bs based on site directed mutagenesis by rational investigation of PCR mutant enzymes. We have reported the mutant enzymes with increased catalytic efficiency[11] and also with increased thermal stability.[12] One of such mutants, Ser231Lys showed significant increase in the thermal stability, moreover, this enzyme retains almost similar catalytic activity as wild type enzyme. Therefore, the application of Ser231Lys for glucose enzyme sensor will enable us to construct an enzyme sensor with increased operational stability.

Here we report two approaches in constructing glucose enzyme sensors with increased thermal stability employing engineered PQQGDH-B. First, we demonstrate the enzyme glucose sensor employing an engineered PQQGDH-B, Ser231Lys, and show its extended thermal stability. Second we report the effect of cross-linking chemical modification on the thermal stability of PQQGDH-B. Utilizing cross-linked PQQGDH-B, we demonstrate the FIA system for glucose measurement.

2 Experimental

2. 1 Reagents

Glucose was obtained from Kanto Chem (Tokyo, Japan), glutaraldehyde was from Kishida Chem (Tokyo, Japan), 1-methoxyphenazinemethosulphate (methoxy-PMS) and ferrocene were from Wako Pure Chemical Industries (Osaka, Japan), carbon paste was from BAS Co. (Indiana, USA). All other reagents were of analytical grade.

2. 2 Enzyme preparation and cross-linking procedure

For the expression of wild type and Ser231Lys, *Escherichia coli* PP2418 was used as the host strain.[13] Purified PQQGDH-Bs was prepared from the cell free extract of the recombinant *E. coli*, as described previously.[12]

Holo-enzyme form of PQQGDH-Bs was obtained by incubation of 15 min at room temperature in 10 mM (M = mol dm^{-3}) MOPS buffer (pH 7.0) solution containing 1 mM $CaCl_2$ and 1 μM PQQ. The cross-linked PQQGDH-B was prepared as follows. Glutaraldehyde solution (final concentration 0.1%) was added in wild type PQQGDH solution (enzyme concentration 30 mg ml^{-1}) and stirred for 30 min at room temperature. And then the sample was dialyzed in 10 mM Tris-HCl buffer (pH 7.0) for overnight at 4℃ in order to remove excess gluteraldehyde and blocking active aldehyde groups by the amino base of Tris.

2. 3 Investigation of thermal stability of cross-linked PQQGDH-B

The residual activity was measured after incubation of each enzyme sample for 10 min at various temperature in 10 mM MOPS buffer (pH 7.0) solution containing 1 mM $CaCl_2$ and 1 μM PQQ. Residual activity at 30℃ was determined and used as the control (100%). The enzyme activity was measured as described previously.[1)]

2. 4 Fabrication of enzyme electrodes

1) Enzyme electrode for batch type

Enzyme electrode employing Ser231Lys or wild type enzyme or cross-linked PQQGDH-B for batch type sensor, was constructed as follows. 25 unit of Ser231Lys or wild type enzyme or cross-linked PQQGDH-B (25 unit of native PQQGDH-B was cross-linked and utilized. 0.167 mg of bovine serum albumin was added to this sample to be immobilized by glutaraldehyde at the electrode) solution and 20 mg of carbon paste was mixed and lyophilized. The end of hole of carbon paste electrode (BAS co. Model No.11-2210, inner diameter 3 mm, and geometric surface area 0.28 cm^2) was filled with the enzyme-carbon paste mixture. The electrode was dipped in a 10 mM MOPS buffer (pH 7.0) solution containing 1% glutaraldehyde and stirred for 30 min at room temperature. After then, the electrode was dipped in 10 mM Tris-HCl buffer (pH 7.0) and stirred for 20 min at room temperature and equilibrated in 10 mM MOPS buffer (pH 7.0). The enzyme electrodes were stored in 10 mM MOPS buffer (pH 7.0) solution containing 1 mM $CaCl_2$ and 1 μM PQQ at 4℃ until the usage. The operation of batch type sensors and cyclic voltammograms measurement were carried out using three electrode system (in water jacket cell: kept on 25℃). An enzyme electrode was used as the working electrode, a Pt wire electrode (0.5 mm diameter, Tanaka noble metal Co., Tokyo Japan) as the counter electrode and an Ag/AgCl electrode (BAS Co.) as the reference electrode. The reaction solution was 10 mM MOPS buffer (pH 7.0) solution containing 1 mM methoxy-PMS as an electron mediator, 1 mM $CaCl_2$ and 1 μM PQQ and the total volume was 10 ml. Cyclic voltammograms were swept between −300 mV to +300 mV vs. Ag/AgCl with a scan rate of 20 mV sec^{-1} by a function generator HB-111 (Hokuto-Denko). Working potential of the steady-state measurement was +100 mV vs. Ag/AgCl controlled by a potentiostat, and the reaction solution was stirred. The current increase following by the injection of 100 μl of a sample solution containing glucose was measured.

2) Enzyme electrode employing cross-linked PQQGDH-B for FIA system

The PQQGDH-B (25 unit of native PQQGDH-B was cross-linked and utilized. 0.167 mg of bovine serum albumin was added) solution and 20 mg of carbon paste was mixed and lyophilized. 6.6 mg of ferrocene as mediator were mixed with the lyophilized mixture. The end of holes of carbon paste electrode (dual type of carbon paste electrode for the flow cell from BAS Co., Model No. 11-1004 inner diameter is 3 mm, and geometric surface area of one hole is 0.28 cm^2) was filled with the enzyme-ferrocene-carbon paste mixture. The electrode was dipped in a 10 mM MOPS buffer (pH 7.0) solution containing 1% glutaraldehyde and stirred for 30 min at room temperature. After then, the electrode was dipped in 10 mM Tris-HCl buffer (pH 7.0) and stirred for 20 min at room temperature and equilibrated in 10 mM MOPS buffer (pH 7.0). The enzyme electrode was used as the working electrode, a stainless tube as the counter electrode and an Ag/AgCl electrode as the reference electrode. These three electrodes were mounted in a cross-flow cell (BAS Co., Model No.11-2456). The amperometric flow cell was connected into a flow injection system (Auto sampler AS-8020 (TOHSO, Japan) with a 20 μl sample loop and a pump). The carrier solution was 10 mM MOPS buffer (pH 7.0) with flow rate at 0.5 ml min^{-1}. The potential was controlled by a potentiostat HA-151 (Hokuto-denko, Tokyo, Japan) and the working potential was +200 mV vs. Ag/AgCl. The peak current following by the injection of 20 μl of a sample solution containing glucose was measured.

3 Results

3. 1 Characterization of cross-linked PQQGDH-B

Fig. 1 shows thermal stability of cross-linked PQQGDH-B. Cross-linked PQQGDH-B showed higher thermal stability than native PQQGDH-B. The significant differences were observed at the thermal inactivation at temperatures higher than 50℃. Since the time course of initial thermal inactivation was describable as the first rate constant equation, we compared the half-lifes of cross-linked PQQGDH-B with that of wild type at 55℃. The half-life of cross-linked PQQGDH-B was 63 min whereas native PQQGDH-B was 4 min. These results suggested that the thermal stability of PQQGDH-B was significantly improved by the cross-linking chemical modification. Since PQQGDH-B is a homo-dimeric enzyme and does not show enzyme activity as the monomeric state, the cross-linking modification might result in intra-subunit cross-linking, prevent the dissociation of the homo-dimer, consequently increase the stability of its quaternary structure. The specific activity for 100 mM glucose of cross-linked PQQGDH-B was 299 U mg $protein^{-1}$, which was about 10% of native PQQGDH-B (2977 U mg $protein^{-1}$ for 70 mM glucose). The retained specific activity of cross-linked PQQGDH-B was however, higher or similar as that for commercially available glucose oxidase.

3. 2 Enzyme electrodes employing Ser231Lys and cross-linked PQQGDH-B

Engineered PQQGDH-B, Ser231Lys was utilized for the glucose sensor. Cyclic voltammograms were carried out in 10 mM MOPS buffer (pH 7.0) solution containing 1 mM methoxy-PMS, 1 μM PQQ and 1 mM $CaCl_2$ at potential range from −300 mV to +300 mV vs. Ag/AgCl with a scan rate of 20 mV sec^{-1} (Fig. 2a). The oxidation and reduction peaks of cyclic voltammogram were appeared at about 0 mV and −250 mV vs. Ag/AgCl, respectively. After the addition of 4.95 mM glucose to the reaction cell, the anodic current increased due to the methoxy-PMS mediated bioelectrocatalytic oxidation of glucose (Fig. 2b). We chose +100 mV vs. Ag/AgCl as the working potential in the following measurement.

The typical response curve of the glucose sensor immobilizing Ser231Lys was shown in Fig. 3a. The sensor reached its steady-state value within 10 sec. The response time was almost the same as that of the glucose sensor on which cross-linked PQQGDH-B was immobilized (data not shown). The difference in the steady-state current after the injection of glucose sample was used as current increase and was plotted against glucose concentrations. The typical calibration curves of the glucose sensor utilized Ser231Lys and cross-linked PQQGDH-B were shown in Fig. 3b. These results show that the dynamic ranges of both sensors for glucose were from 10 μM to 3 mM. The difference of the sensor responces between the two glucose sensors depended on the activity of the enzymes.

Thermal stabilities of enzyme electrodes immobilizing Ser231Lys and cross-linked PQQGDH-B were investigated. After the heat treatment of enzyme electrode at 60℃, a sensor response for 0.99 mM glucose was measured at 25℃ (Fig. 4). The electrodes employed Ser231 Lys and cross-linked PQQGDH-B retained about 80% of

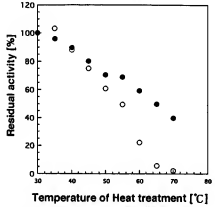


Fig. 1 Thermal stability of cross-linked PQQGDH-B. After heat treatment at various temperatures for 10 min, residual activity was measured (●: cross-linked PQQGDH-B and ○: Wild type). Enzyme activity after heat treatment at 30℃ for 10 min was used as the controll (100%).

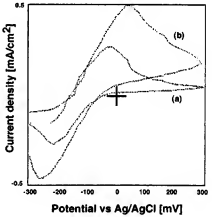


Fig. 2 Cyclic voltammograms of the sensor employing Ser 231Lys in 10 mM MOPS buffer (pH 7.0) solution containing 1 mM methoxy-PMS, 1 μM PQQ, 1 mM $CaCl_2$. a) in the absence of glucose and b) in the presence of glucose. Scan rate was 20 mV sec^{-1}.

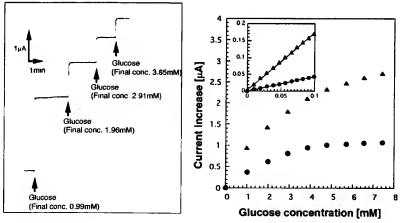


Fig. 3 Response curve of the sensor employing Ser231Lys (a) and calibration curves of the sensor employing Ser231Lys(▲) and cross-linked PQQGDH-B(●). (b) The measurement was carried out in 10 mM MOPS buffer (pH 7.0) solution containing 1 mM methoxy-PMS, 1 μM PQQ and 1 mM $CaCl_2$. The operating potential: +100 mV vs. Ag/AgCl, Temperature for measurement: 25℃.

initial activity after heat treatment at 60℃ for 2 hours, whereas the electrode employing wild type enzyme decreased to 30%. The results show the enzyme electrode employed Ser 231 Lys and cross-linked PQQGDH-B retained high thermal stability.

3. 3 Application of cross-linked PQQGDH-B for FIA glucose sensing system

We constructed a Flow Injection Analysis (FIA) system with an electrode employing cross-linked PQQGDH-B. After 1 min of injection, the peak current appeared, and within 5 min (Fig. 5a), the base current recovered. The response obtained from this FIA system was lower than that obtained from batch type sensor. This might be mainly due to the difference in the electron mediator utilized in the electrodes. In the batch type sensor, methoxy-PMS was utilized, and ferrocene was utilized in FIA system. The electron transfer between PQQGDH-B and ferrocene was not rapid as that with methoxy-PMS. However, the application of water insoluble ferrocene in carbon paste electrode enable us to construct an enzyme electrode system which does not require additional electron mediator in a carrier solution for the construction of FIA system. The calibration curve of this system is shown in Fig. 5b. The dynamic range of this system was from 1 mM to 50 mM.

The operational stability of FIA system was investigated by injecting 20 mM glucose every 10 min for 100 times (1000 min), consecutively (Fig. 6). After 1000 min of operation, no decrease in the sensor response was observed. The SD was within 3% during this period of sensor operation. These results suggested that FIA system employing cross-linked PQQGDH-B was stable for more than 100 time consecutive measurements. The FIA system employing native PQQGDH-B was previously reported.[10] The half life time of the reported sensor system was 40 hours. Considering that native PQQGDH-B was not stable in a solution, the stable FIA system will not be achieved using native enzyme. Cross-linking modification of PQQGDH-B and its application for FIA system showed a novel potential of PQQGDH-B for not only in the field of FIA system, but also for the long term continuously operating system, such as for implanting sensor system.

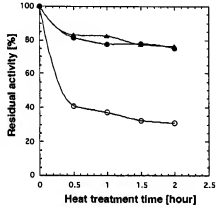


Fig. 4 Thermal stability of the sensor employing Ser231Lys and cross-linked PQQGDH-B. After heat treatment at 60℃, responses for 0.99 mM glucose were measured at 25℃ (▲: Ser231Lys, ●: cross-linked PQQGDH-B, and ○: Wild type). All other conditions were the same as for Fig. 3.

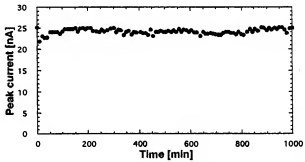
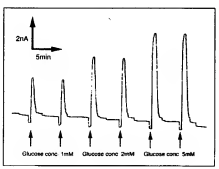


Fig. 6 Operational stability of the sensor employing cross-linked PQQGDH-B in a FIA system for the injections of 20 mM glucose every 10 min. Measurement condition was the same as for Fig. 5.



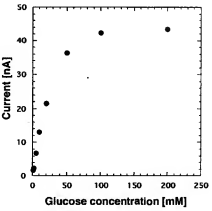


Fig. 5 Response curve (a) and Calibration curve (b) of the sensor employing cross-linked PQQGDH-B. Carrier solution: 10 mM MOPS buffer (pH 7.0). Flow rate: 0.5 ml min^{-1}, Injection volume: 20 μl, The operating potential: +200 mV vs. Ag/AgCl, Temperature for measurement: room temperature.

4 Discussion

This paper showed two different approaches in constructing thermostable glucose enzyme sensor based on water soluble PQQGDH. We utilized a protein engineered PQQGDH-B, Ser231Lys and a cross-linked PQQGDH-B. Utilizing these engineered enzymes, glucose sensors with high operational stability can be constructed, which will not be achieved by using wild type enzyme. These results suggested the further expansion of the utilization of PQQGDH-B. The use of stable PQQGDH-B may allow the designing in the enzyme sensor fabrication process regardless of the fragile properties of native enzyme. Moreover, PQQGDH-B can be used not only for the disposable type sensor, but also as the component for the enzyme sensors, of which repeated operations are expected, such as for FIA analyses and also for the implantable glucose sensors. Ser231Lys was constructed based on our rational site directed mutagenesis. Recently, the 3D structure of PQQGDH-B was elucidated.[15] On the basis of its 3D structure, Ser 231 is revealed not to be involved in the amino acid residues constructing dimer interface. Therefore, Ser231 may have a significant role in structural stability of β-propeller fold of each subunit, as we previously assumed as the role of Ser231 based on predicted topological structure.[10] On the other hand, the cross-linking chemical modification of PQQGDH-B was aimed to stabilize the quaternary structure of PQQGDH-B. Since PQQGDH-B have 39 lysine residues, about 10% of whole residues, the cross-linking event by glutaraldehyde resulted in both intra subunit and inter subunit. Gel chromatography analysis of cross-linked PQQGDH-B revealed that the major fraction showing PQQGDH activity was eluted at the MW about 100 kDa (results was not shown). This result indicated that under our experimental condition, cross-linking modification mainly resulted in the inter subunit cross-linking. Therefore, the thermal stability of cross-linked PQQGDH-B was mainly due to the stabilization of quaternary structure. The cross-linking modification of enzymes and consequent stabilization of enzymes using glutaraldehyde has been reported by many researchers. This method is simple but most of the cases, decrease in the catalytic activity associated. The cross-linking modification of PQQGDH-B was also in the case. Thus constructed cross-linked PQQGDH-B showed similar or higher thermal stability than Ser231 Lys enzyme. In spite of significant increase in the thermal stability, however, catalytic activity decreased to 10% of the native enzyme. These results indicated that cross-linking modification might be also occurred at the amino acid residues responsible for the catalytic activity. Considering the principle of chemical modification by glutaraldehyde, site directed modification is impossible, therefore, such a negative effect is inevitable. Further engineering approach in focusing in the stabilization of quaternary structure of PQQGDH-B is, therefore, being expected.

References

1) G. J. Kost, H. -T. Vu, J. H. Lee, P. Bourgeois, F. L. Kiechle, C. Martin, S. S. Miller, A. O. Okorodudu, J. J. Podczasy, R. Webster, and K. J. Whitlow, *Crit. Care. Med.*, **26**, 581 (1998).
2) B. Schmidt, *Clinica. Chim. Acta.*, **266**, 33 (1997).
3) K. Sode and H. Sano, *Biotechnol. Lett.*, **16**, 455 (1994).
4) K. Sode, K. Watanabe, S. Ito, K. Matsumura, and T. Kikuchi, *FEBS Lett.* **364**, 325 (1995).
5) K. Sode, H. Yoshida, K. Matsumura T. Kikuchi, M. Watanabe, N. Yasutake, S. Ito, and H. Sano, *Biochem. Biophys. Res. Commun.*, **211**, 268 (1995).
6) K. Sode and K. Kojima, *Biotechnol. Lett.*, **19**, 1073 (1997).
7) K. Sode and H. Yoshida, *Denki Kagaku* (presenstly *Electrochemistry*), **65**, 444 (1997).
8) A. B. Witarto, T. Ohtera, and K. Sode, *Appl. Biochem. Biotechnol.*, **77-79**, 159 (1999).
9) H. Yoshida, K. Kojima, A. B. Witarto, and K. Sode, *Protein Eng.*, **12**, 63 (1999).
10) K. Sode, T. Shimakita, S. Ohuchi, and T. Yamazaki, *Biotech. Lett.*, **18**, 997 (1999).
11) S. Igarashi, T. Ohtera, H. Yoshida, A. B. Witarto, and K. Sode, *Biochem. Biophys. Res. Commun.*, **264**, 820 (1999).
12) K. Sode, T. Ohtera, M. Shirahane, A. B. Witarto, S. Igarashi, and H. Yoshida, *Enzyme and Microbial Technology*, in press.
13) A. M. Cleton-Jansen, N. Gossen, O. Feyet, and P. ven de Putte, *J. Bacteriol.*, **172**, 6308-6315 (1990).
14) L. Ye, M. Hammerle, A. J. J. Olsthoorn, W. Schuhmann, H. -L. Schmidt, J. A. Duine, and A. Heller, *Anal. Chem.*, **65**, 238 (1993).
15) A. Oubrie, H. J. Rozeboom, K. H. Kalk, J. A. Duine, and B. W. Dijkstra, *J. Mol. Biol.*, **289**, 319 (1999).